Two Loci Exert Major Effects on Chlorogenic Acid Synthesis in Maize Silks

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ABSTRACT

Chlorogenic acid (CGA) in maize (*Zea mays* L.) silks has been implicated in resistance to corn earworm (*Helicoverpa zea* Boddie) with a mechanism similar to the flavone, maysin. However, the genetic basis of CGA synthesis is poorly understood. Our goal was to identify candidate loci affecting the biosynthesis of CGA using quantitative trait locus (QTL) analyses on three F_2 populations: (A619 × Mp708)F_2, (A619 × Mo6)F_2, and (Mo6 × Mp708)F_2. Chlorogenic acid and flavone contents in silks were measured, linkage maps generated, and significant loci identified with composite interval mapping (CIM) and ANOVA multiple-effects models for the three populations. Of the QTLs detected, two exerted major effects; one corresponding to the *p1* locus, and the other a novel locus we named *qtl2*. The main effect of the functional allele at the *p1* locus was to increase both CGA and flavones, while the positive allele at the *qtl2* locus only increased CGA. An epistatic interaction between *p1* and *qtl2* alleles in the (A619 × Mo6)F_2 population caused an increase in CGA with a concomitant decrease in flavones. The rare ability of Mo6 and Mp708 to synthesize CGA in excess of flavones is primarily a result from the effects at the *qtl2* locus.

Chlorogenic acid is a secondary metabolite synthesized in many plants (Clifford, 1999; Herrmann, 1989). Chlorogenic acid is implicated in free radical scavenging (Chen and Ho, 1997), inhibition of lipid peroxidation (Ohnishi et al., 1994), enzymatic browning of fruits and vegetables (Walker, 1995), antifungal activity (Maher et al., 1994), and host-plant resistance against insects (Dowd and Vega, 1996; Isman and Duffey, 1982). Of particular interest to our group are the activities of CGA on corn earworm. The corn earworm is a polyphageous lepidopteran pest that reduces fertility of maize and provides entry points for ear rotting fungi and other pathogens by feeding on silks and kernels. Host-plant resistance to corn earworm was detected in maize germplasm with high levels of maysin, a C-glycosylflavone (Elliger et al., 1980a), and further analysis of maysin and other flavones suggested a requirement for adjacent phenolic hydroxyl groups in the molecule to confer resistance (Elliger et al., 1980b). Upon ingestion by corn earworm larvae, these adjacent hydroxyls are oxidized to quinones that sequester available amino acids from digestion, cause oxidative stress, reduce larval weight, and lengthen days to pupation (Hurrell et al., 1982; Summers and Felton, 1994; Wiseman and Carpenter, 1995). Chlorogenic acid has a similar adjacent hydroxyl ring structure as maysin, and has also been implicated in growth-reduction of lepidopterans (Duffey and Stout, 1996; Isman and Duffey, 1982).

Chlorogenic acid is a product of the phenylpropanoid biochemical pathway, and shares substrates with lignin and flavonoid branch pathways (Fig. 1). The first step in the phenylpropanoid pathway involves the deamination of phenylalanine to cinnamate via the enzyme phenylalanine ammonia lyase (PAL). Phenylalanine ammonia lyase RNA levels respond to several regulatory signals (Costa e Silva et al., 1993; Christie et al., 1994), and were reported to be a main factor affecting CGA levels in transgenic systems (Bate et al., 1994). Synthesis of CGA from cinnamate requires four enzymes to complete two hydroxylations on the aromatic ring and a conjugation of the hydroxycinnamate and quinate moieties: cinnamate-4-hydroxylase (C4H), 4-coumaroyl-CoA ligase (4CL), coumaroyl-CoA:quinate hydroxycinnamoyltransferase (CQT), and coumarate/coumaroylquinate-3-hydroxylase (C3H). The order of events can vary among plant systems, but previous studies have suggested the pathways shown in Fig. 1 (Kuhnl et al., 1987; Rhodes and Wooltorton, 1976; Ulbrich and Zenk, 1979).

Our objective was to identify loci affecting the biosynthesis of CGA using QTL analyses, and identify candidate genes from the phenylpropanoid biosynthetic pathway that could be responsible for those loci. Previous QTL studies have detected loci and genes with major effects for maysin (McMullen et al., 1998), but populations specifically designed to address the synthesis of CGA have not been analyzed. We utilized three F_2 populations from three maize inbred lines allowing a circular comparison of QTLs. The inbred parents of two populations differ in their ability to synthesize CGA in the silk tissue, and the third population was constructed from two high CGA lines. Linkage disequilibrium and practical limitations on population sizes generate QTL confidence intervals of approximately 10 centimorgans (cM) (Darvasi et al., 1993). Chromosomal regions of this map length in maize contain approximately 300 genes complicating identification of specific genes controlling quantitative traits. However, information available about the enzyme steps in the phenylpropanoid pathway necessary for CGA synthesis, and map locations of several genes involved, provide plausible and testable candidate genes for some QTLs.

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Abbreviations: 4CL, 4-coumaroylCoA ligase; C3H, coumarate-3-hydroxylase; C4H, cinnamate-4-hydroxylase; CGA, chlorogenic acid; CHS, chalcone synthase; CIM, composite interval mapping; CQT, coumaroylCoA:quinate hydroxycinnamoyl transferase; LOD, log-odds ratio; PAL, phenylalanine ammonia lyase; QTL, quantitative trait locus; SSR, simple sequence repeat.

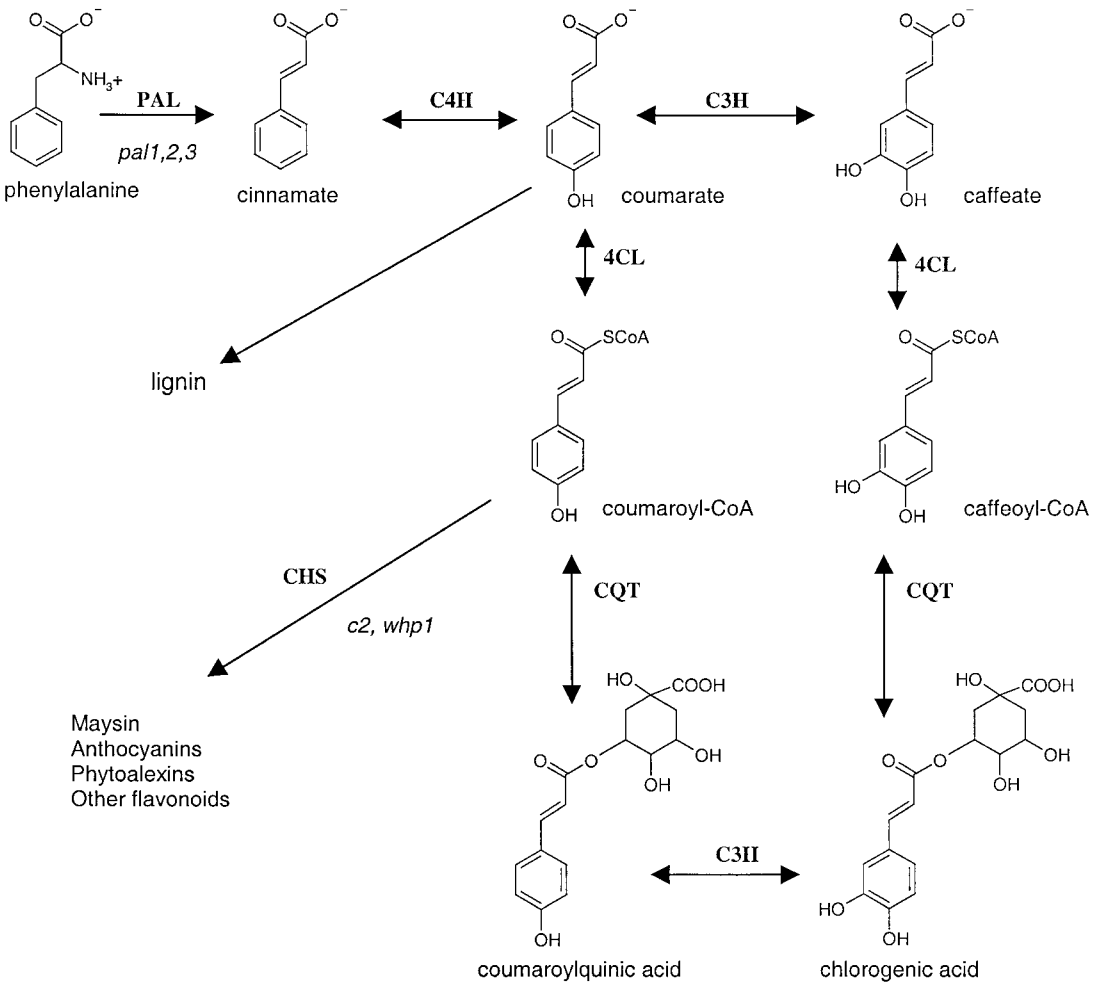

Fig. 1. The general phenylpropanoid biochemical pathway and proposed steps leading to CGA. Enzymes are in black caps and genes in italics. PAL, phenylalanine ammonia lyase, C4H, cinnamate-4-hydroxylase; C3H, coumarate-3-hydroxylase; 4CL, 4-coumaroylCoA ligase; CQT, coumaroylCoA:quinate hydroxycinnamoyl transferase; CHS, chalcone synthase. This sequence of events is based on preferred substrates and time-course studies in other plants, and branchpoints into lignin and flavonoid biosynthesis are shown.

MATERIALS AND METHODS

Mapping Populations

The F_2 populations were generated from three maize inbred lines: A619, Mo6, and Mp708. Mp708 and Mo6 synthesize high amounts of CGA (Fig. 2), in excess of maysin, a C-glycosyl flavone typically the most abundant, extractable phenylpropanoid product in silks of most field maize varieties. Mo6 was developed from the Tennessee golden prolific line T8 at the Missouri Agricultural Experiment Station, Columbia, MO. Mo6 synthesizes high levels of CGA and moderate amounts of isoorientin, a C-glucosylflavone. Mp708 was derived from selfed selections for resistance to southwestern corn borer (*Diatraea grandiosella* Dyar) and fall armyworm (*Spodoptera frugiperda* Smith) from the cross of Mp464 × Tx601 at the Mississippi Agriculture and Forestry Experiment Station, Starkville, MS. Mp708 produces high amounts of CGA and moderate amounts of maysin, a C-glycosyl flavone. A619 was developed from (A171 × Oh43) × Oh43 at the Minnesota Agricultural Experiment Station, St. Paul, MN. A619 accumulates negligible levels of CGA and flavones in the silk tissue (Fig. 2). The populations (A619 × Mp708)F_2, (A619 × Mo6)F_2, (Mo6 × Mp708)F_2 included 427, 348, and 190 individuals, respectively. Each F_2 population was derived from two F_1 ears, and was grown at the University of Missouri Agronomy Research Center, Columbia, MO, along with parent and F_1 plants. The population (A619 × Mp708)F_2 was grown during the summer of 1998 while the other two populations were grown in 1999. All three populations were grown under standard agronomic practices for our region.

Phenotypic Analysis

For all populations, primary ear shoots were covered before silk emergence. Silks were collected two days after emergence, weighed, and lyophilized. Lyophilized silks were analyzed for flavone and CGA contents by reverse-phase HPLC (Snook et al., 1989, 1993). Parental and F_1 phenotype values represent a mean of 5 to 15 individuals. Phenotype analyses of (A619 × Mo6)F_2 and (Mo6 × Mp708)F_2 populations were conducted at the Richard B. Russell Research Center, University of Georgia, Athens, GA, and from (A619 × Mp708)F_2 at the National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL. The amount of CGA and flavones were determined and reported as the percent fresh weight of the silk.

Genotype Analysis

Leaf tissue was harvested from parental, F_1, and F_2 plants at the mid-whorl stage, lyophilized, and DNA was extracted from lyophilized tissue. Genotypes were determined with simple sequence repeat (SSR) markers. The DNA extraction procedures

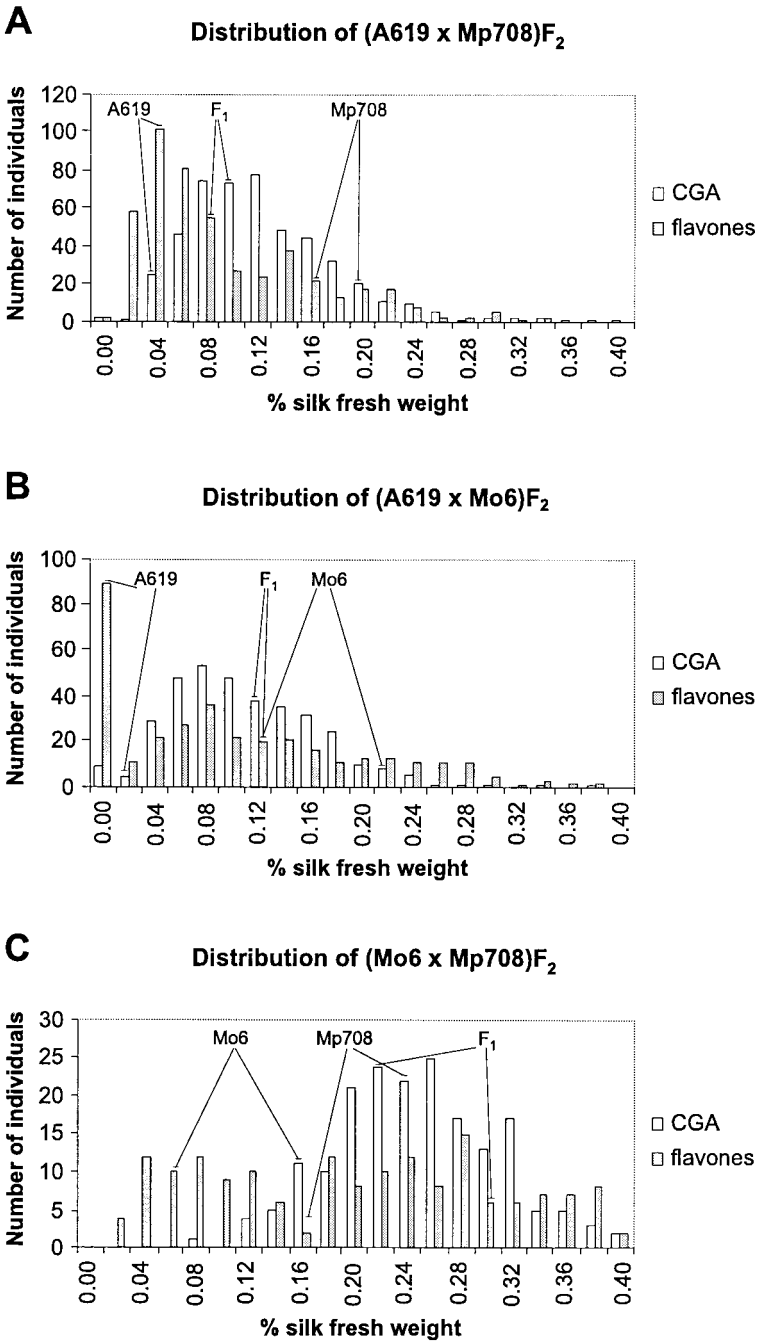

Fig. 2. Distribution of CGA and flavone values from individuals of A: (A619 × Mp708)F_2, B: (A619 × Mo6)F_2, and C: (Mo6 × Mp708)F_2 populations. The percent fresh silk weight of each chemical (CGA or flavones) was measured with reverse-phase HPLC. Parental and F_1 values are shown for each population, and represent the means of 5-15 individuals.

and SSR reaction conditions are available at MaizeDB, www.agron.missouri.edu/ssr.html, verified April 3, 2002. Amplification products were resolved on 4 to 5% (w/v) agarose gels at 115 V, and viewed with the Eagle-Eye *uv* detection system (Stratagene, La Jolla, CA). In some genome areas where polymorphism was limited and agarose gels could not resolve differences, fluorescently-labelled SSR primers were used to generate polymorphic PCR products that were analyzed by the ABI3700 (PE Biosystems, Foster City, CA) DNA sequencer system. Amplification products were diluted and resolved through capillary electrophoresis and the size of the SSR product determined relative to an internal molecular weight marker, as analyzed with GENESCAN software (PE Biosystems, Foster City, CA).

Statistical Analysis

Linkage maps were generated by MAPMAKER/EXP, version 3.0 (Whitehead Institute, Cambridge, MA), on the basis of a minimum LOD score of 3.0 and a maximum linkage distance of 50 cM. Segregation distortion was tested by Chi-square analysis with $P < 0.01$. The normality of F_2 population distributions was tested by the Shapiro-Wilk procedure of PROC UNIVARIATE, SAS system (SAS Inst., 1999). Marker-

Table 1. Composite interval mapping location, significance, effects, and candidate genes of QTLs for CGA and flavones from three maize populations.

Trait	Chrom./bin†	Nearest marker	Max. LOD‡	R^2§	Gene Effect¶		Candidate gene
					Add.	Dom.	
A619 × Mp708							
CGA	1.03	*umc1021*	23.09	19.9	−0.035	0.023	*p1*
	2.06	*umc1065*	8.94	7.9	−0.025	0.004	*qtl2*
Flavone	1.03	*umc1021*	39.98	36.3	−0.068	−0.024	*p1*
	4.01	*umc1164*	3.26	3.0	−0.001	−0.028	
	7.03	*bnlg1070*	4.26	3.3	0.024	−0.002	
	9.02	*umc1037*	3.95	3.5	−0.023	−0.004	*rem1*
A619 × Mo6							
CGA	1.03	*bnlg182*	16.40	13.0	−0.025	0.026	*p1*
	2.06	*bnlg1138*	25.72	22.7	−0.039	0.000	*qtl2*
	3.05	*bnlg1113*	3.69	2.9	0.015	0.000	
	4.06	*bnlg1621*	11.30	9.8	0.028	0.003	
	5.05	*mmc0282*	5.51	3.7	−0.016	−0.004	*pal*
Flavone	1.02	*bnlg1007*	55.64	49.7	−0.010	0.000	*p1*
	1.10	*umc1431*	3.59	2.3	0.021	−0.005	
	2.06	*bnlg1138*	3.12	2.4	0.019	0.021	*qtl2*
	2.08	*bnlg1606*	7.50	7.7	0.028	0.040	*sm2*
	5.07	*bnlg1346*	4.12	4.3	0.027	−0.018	
	9.02	*umc1037*	5.56	4.1	−0.029	0.005	*rem1*
Mo6 × Mp708							
CGA	1.06	*bnlg1057*	5.04	7.0	−0.025	0.001	
	2.08	*bnlg1267*	10.70	14.5	−0.033	0.027	*sm2*
	3.06	*dupssr23*	4.44	7.5	−0.026	−0.013	
	4.08	*umc1132*	4.23	6.1	−0.024	−0.005	*c2*
	7.05	*phi082*	4.65	6.5	0.025	−0.010	
Flavone	1.09	*phi094*	4.51	6.5	0.053	−0.020	
	2.08	*bnlg1267*	22.75	34.1	−0.114	0.103	*sm2*
	3.06	*dupssr23*	4.23	8.0	−0.069	−0.011	
	6.01	*y1ssr*	3.69	9.6	−0.068	−0.033	

† Bins are approximately 20 cM subdivisions of each chromosome and are represented on the right hand side of the decimal. Thus chromosome/bin 1.03 represents chromosome 1, bin 3.
‡ LOD = log of the maximum likelihood ratio with H_0: no additive or dominance effects.
§ R^2 = the partial R^2, or percent of phenotypic variance explained by that QTL.
¶ Gene effects are the deviation from the mid-parent, with negative values representing the maternal parent on the left-hand side of the cross.

trait associations affecting CGA, isoorientin, maysin, and total flavone (maysin + isoorientin) were detected by composite interval mapping (CIM) and multiple-effects ANOVA models. Composite interval mapping was performed with QTL Cartographer, Version 1.14a (Basten et al., 1994, 2000), and markers used as parameters were selected from a forward/backward stepwise regression with thresholds for inclusion and exclusion at $P \leq 0.01$. For each population and trait, we determined empirical threshold levels for declaring QTL significance at a genome-wise Type I error rate of 0.05 (Churchill and Doerge, 1994). Positional relationship of QTLs from different populations was based on location and genetic distance between markers shared by the populations. Multiple effect models were generated with PROC GLM in SAS from markers significant in stepwise regression, and from digenic interactions significant in EPISTACY, a two-way ANOVA procedure (Holland, 1998). Markers with main effects were initially identified by stepwise forward/backward selection ($P \leq 0.01$), and inclusion in the final model required a $P \leq 0.05$ for the Type III sums of squares (SAS Inst., 1999). All digenic interactions for markers were initially identified with EPISTACY ($P \leq 0.001$), and resulting significant interactions were included in the final model if $P \leq 0.05$ for the Type III sums of squares. Genotype class means were obtained through PROC MEANS using the CLASSES statement of SAS software.

RESULTS

Phenotypic Data

Both Mo6 and Mp708 synthesized large amounts of CGA, in excess of flavones (Fig. 2). Mo6 accumulated isoorientin instead of maysin; but when crossed to A619 or Mp708, isoorientin was replaced by maysin in the F_1. To simplify analysis, we have summed isoorientin and maysin, and reported that sum as *flavones*. A619 synthesized negligible amounts of both CGA and flavones. In the (A619 × Mp708)F_2 and (A619 × Mo6)F_2 populations both CGA and flavone distributions exhibited transgressive segregation tails of high values (Fig. 2). Additionally, approximately one third of the (A619 × Mo6)F_2 progeny synthesized no flavones, resulting in a bimodal distribution for that trait. The (Mo6 × Mp708)F_2 population distribution for CGA was normal ($P = 0.2954$), but displayed a nonnormal, broad distribution of values for flavones. The phenotype means of CGA and flavones in this population were approximately equivalent to the Mp708 value, and transgressive segregant plants exceeded parental CGA and flavone values up to two-fold.

Linkage Maps

Framework maps of 10 linkage groups were generated for all three populations. For the (A619 × Mp708)F_2 population, 91 SSR markers were anchored onto a map for a total genetic distance of 1570 cM. For the (A619 × Mo6)F_2 population, 93 SSR markers were anchored onto a map representing a total genetic distance of 1486 cM. For the (Mo6 × Mp708)F_2 population, 91 SSR markers were anchored onto a map representing a total

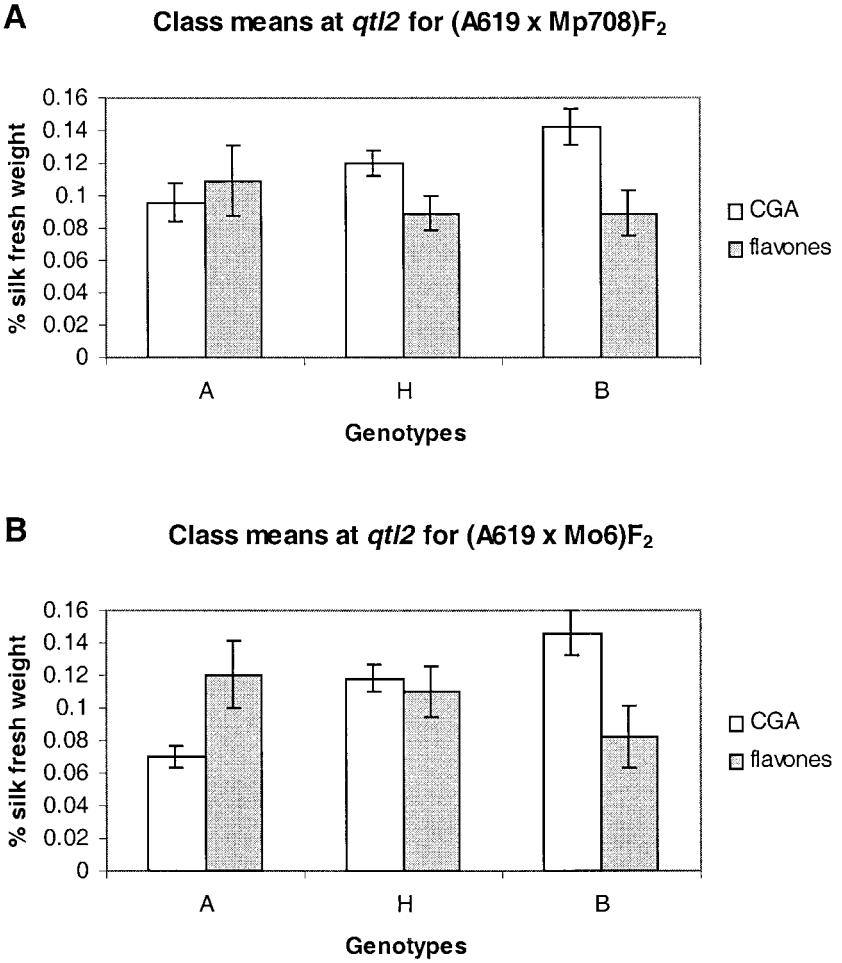

Fig. 3. Genotype class means for CGA and flavones at the marker genotype nearest the *qtl2* locus in A: (A619 × Mp708)F_2 and B: (A619 × Mo6)F_2 populations. Genotype A, homozygous A619 alleles; H, heterozygous; and B, homozygous Mp708 or Mo6 alleles depending upon the population. Standard error bars are taken to the second standard deviation.

genetic distance of 1353 cM. Polymorphism on the tip of the long arm of chromosome 7 in the (Mo6 × Mp708)F_2 population could not be detected. In this population the marker *bnlg1036* was located within 9.7 cM of *phi127*, but the SSR consensus map places these two markers in bins 2.05 and 2.08, respectively, approximately 30 cM apart. Except for this locus, marker order and approximate distances in all three populations was consistent with the SSR consensus maps in MaizeDB, www.agron.missouri.edu. Marker loci spanning bins 4.00-4.03 showed segregation distortion in the (A619 × Mp708)F_2 population and markers from 4.00-4.07 were distorted in the (Mo6 × Mp708)F_2 population. The genetic maps for all three populations will be made available on the MaizeDB.

QTL Detection

(A619 × Mp708)F_2

For CGA synthesis, two QTLs were detected by CIM (Table 1). The QTL with the largest effects was located on chromosome 1 in bin 1.03 near *umc1021*. The 10 maize chromosomes are subdivided into bins spaced approximately 20 cM apart, with the bin denoted on the right-hand side of a decimal and the chromosome on the left-hand side (Gardiner et al., 1993; Davis et al., 1999). Quantitative trait loci confidence intervals generally span 10 to 20 cM, so we describe QTL location by bin. We ascribed the QTL detected in bin 1.03 to the *pericarp color1* (*p1*) gene from Mp708, which has a *p1-wwb* (white pericarp, white cob, browning silks) allele. The second major QTL was located near marker *umc1065* in bin 2.06, and will be referred to as *qtl2* in this report because no candidate gene from the pathway is known. The *qtl2* locus explained 7.9% of the phenotypic variation, increasing CGA levels by approximately 50% (Fig. 3). An ANOVA multiple-effects model identified five markers as main effects and three digenic interactions, accounting for 43.8% of the total phenotypic variation (Table 2). Both QTLs detected from CIM were included as main effects, as were markers in bins 4.08, 5.00, and 6.01. *bnlg381* and *phi036* were not significant as main effects, but were included in significant interactions.

Four QTLs were detected for flavone synthesis by CIM. The *p1* locus near *umc1021* is a candidate for the effects of the QTL in bin 1.03, and accounted for 36.3% of the phenotypic variation. The *recessive enhancer of maysin* (*rem1*) candidate gene was located near the QTL in bin 9.02 (Byrne et al., 1996), but we have no

Table 2. Multiple-effects models for CGA and flavone accumulation in maize silks from the three populations used in this study.

Population	CGA			Flavone		
A619 × Mp708						
Marker†	**bin**	***P***		**Marker**	**bin**	***P***
p1‡	1.03	0.0000		*p1*	1.03	0.0000
qtl2	2.06	0.0000		*phi021*	4.03	0.0003
phi077	6.01	0.0003		*umc1037*	9.02	0.0077
umc1240	5.00	0.0048		*umc1001*	7.03	0.0101
phi092	4.08	0.0230		*phi077*	6.01	0.0132
bnlg381	2.03	0.0520		*umc1412*	7.04	0.0233
p1 × *bnlg381*	1.03 × 2.03	0.0005		*umc1240*	5.00	0.0810
phi036	3.04	0.0796		*p1* × *umc1240*	1.03 × 5.00	0.0054
phi092 × *phi036*	4.08 × 3.04	0.0082		*mmc0271*	2.07	0.0721
p1 × *umc1240*	1.03 × 5.00	0.0220		*p1* × *mmc0271*	1.03 × 2.07	0.0124
R^2 = 43.8				R^2 = 57.1		
A619 × Mo6						
Marker	**bin**	***P***		**Marker**	**bin**	***P***
p1	1.03	0.0000		*p1*	1.03	0.0000
qtl2	2.06	0.0000		*umc1037*	9.02	0.0000
bnlg1621	4.06	0.0000		*sm2*	2.08	0.0003
umc1173	4.09	0.0000		*bnlg1346*	5.07	0.0011
mmc0282	5.05	0.0000		*umc1295*	7.04	0.0011
umc1478	5.01	0.0003		*bnlg1247*	7.02	0.0022
bnlg1113	3.05	0.0004		*umc1431*	1.10	0.0037
umc1152	10.01	0.0008		*bnlg1927*	4.07	0.0049
sm2	2.08	0.0022		*umc1506*	10.04	0.0092
umc1282	1.01	0.0173		*bnlg2277*	2.02	0.0163
p1 × *qtl2*	1.03 × 2.06	0.0000		*qtl2*	2.06	0.0337
umc1023	6.00	0.3445		*p1* × *umc1431*	1.03 × 1.10	0.0245
mmc0282 × *umc1023*	5.05 × 6.00	0.0000		*p1* × *bnlg2277*	1.03 × 2.02	0.0500
phi260485	7.05	0.3489		*p1* × *qtl2*	1.03 × 2.06	0.0125
umc1023 × *phi260485*	6.00 × 7.05	0.0007		R^2 = 68.0		
sm2 × *bnlg1621*	2.08 × 4.06	0.0008				
qtl2 × *umc1152*	2.06 × 10.01	0.0201				
nc103	6.05	0.5086				
qtl2 × *nc013*	2.06 × 6.05	0.0396				
R^2 = 71.5						
Mo6 × Mp708						
Marker	**bin**	***P***		**Marker**	**bin**	***P***
sm2	2.08	0.0000		*umc1736*	2.09	0.0008
umc1489	3.07	0.0001		*sm2*	2.08	0.0013
c2	4.08	0.0008		*bnlg1523*	3.03	0.0024
umc1019	5.06	0.0068		*y1ssr*	6.01	0.0035
umc1799	7.06	0.0285		*bnlg1057*	1.06	0.0049
umc1153	5.09	0.2281		*bnlg1651*	8.05	0.0363
c2 × *umc1153*	4.08 × 5.09	0.0008		*bnlg1092*	2.01	0.5744
R^2 = 70.7				*sm2* × *bnlg1092*	2.08 × 2.01	0.0060
				c2 × *umc1736*	4.08 × 2.09	0.0060
				R^2 = 83.6		

† Main effects were initially selected from stepwise forward/backward selection with a Type I error rate of 0.01. Interactions were initially identified with EPISTACY, with a Type I error rate of 0.001. Markers and interactions retained in the final model were significant at type III sums of squares of $P < 0.05$.
‡ For markers that could be explained by gene loci, the locus name is noted rather than the marker name.

candidates for the QTLs in bins 4.01 and 7.03. The QTL in bin 4.01 was also flanked by markers exhibiting segregation distortion. A multiple-effects model for flavone synthesis of six main effects and two digenic interactions explained 57.1% of the phenotypic variation. All four QTLs identified from CIM, and a locus in bin 6.01, were included as main effects.

(A619 × Mo6)F_2

Several QTLs were detected for CGA in this population by CIM, including two analogous to those identified in (A619 × Mp708)F_2. The QTL in bin 1.03 included the locus for the *p1-wrb* (white pericarp, red cob, browning silks) allele from Mo6, and accounted for 13% of the phenotypic variation. The QTL in bin 2.06 had the largest effect in this population, explaining 22.7% of the variation for CGA, and its confidence interval was consistent with that of the *qtl2* locus in the first population. Within bin 5.05, the QTL confidence interval included a putative *phenylalanine ammonia lyase1* (*pal1*) gene location. For the QTLs detected in bins 3.05 and 4.06, we could assign no candidate genes. Interestingly, A619 alleles increased CGA at these QTLs. A multiple-effects model for CGA from this population included 10 markers as main effects and six digenic interactions, representing 71.5% of the variation. Nearly all QTLs detected by CIM, and markers in bins 1.01, 2.08, 4.09, 5.01, and 10.01 were included in the model. The QTL in bin 4.06 was not a main effect despite its moderately large effects on the phenotype detected with CIM. Of the six digenic interactions, three included the *qtl2* locus, including the interaction of *p1* and *qtl2*.

Six QTLs were detected for flavone synthesis by CIM. The *p1* locus is a candidate for the QTL in bin 1.02, and

accounted for half of the phenotypic variance. Unlike Mp708, two QTLs for flavones were detected on chromosome 2 in this population. The location of the QTL in bin 2.06 was consistent with the location of the *qtl2* locus, and the location and effects of the QTL in bin 2.08 was consistent with the action at the *salmon silk2* (*sm2*) locus. A locus near *umc1037* in bin 9.02 was also detected in this population in a similar location to the analogous QTL found in the first population. No plausible candidate gene was identified for the QTLs in bins 1.10 and 5.07, where A619 alleles increased flavones. The multiple-effects model for flavone synthesis included 11 markers and three digenic interactions, explaining 69% of the variation. Along with the six QTLs, markers in bins 2.02, 4.07, 7.02, 7.04, and 10.04 were also significant.

(Mo6 × Mp708)F_2

This cross involved two high CGA producing parents and completed a three-way circle of F_2 populations. For CGA synthesis, neither the *p1* nor *qtl2* locus, which exerted the largest effects in the two predecessor populations, was significant in this population. The location of the QTL detected on chromosome 2 in this population was consistent with the *sm2* location, rather than the location of *qtl2*. The *colorless2* (*c2*) gene, encoding the enzyme chalcone synthase, was located within the confidence interval of the QTL in bin 4.08, but markers in that region exhibited segregation distortion. A multiple-effects model in this population included five markers and one digenic interaction to account for 70.7% of the phenotypic variation. Quantitative trait loci detected through CIM in bins 2.08, 3.06, 4.08, and 7.05 were also significant main effects, as was a marker in bin 5.06.

For flavone synthesis, four QTLs were detected by CIM. The location of the most significant QTL, in bin 2.08, was the same position as the *sm2* candidate locus for CGA. No candidate genes could be identified for the QTLs in bins 1.09, 3.06, or 6.01. A multiple-effects model for flavones included five main effect markers and two digenic interactions to explain 83.5% of the phenotypic variation. Interestingly, only QTLs in bins 2.08 and 6.01 detected by CIM were also significant in multiple-effects models, while markers in bins 1.06, 3.03, and 8.05 were specific for the latter.

DISCUSSION

The *p1* Locus

In the (A619 × Mp708)F_2 and (A619 × Mo6)F_2 populations, the *p1* locus showed large effects on both CGA and flavones (Table 1). Mp708 and Mo6 each contained different *p1* alleles that expressed in silks, *p1-wwb* and *p1-wrb*, respectively. The A619 (*p1-www*) allele is nonfunctional for silk expression. P1 is a *Myb*-domain transcription factor known to activate genes in the flavonoid biochemical pathway downstream of CGA synthesis (Grotewold et al., 1994), but a basis for its effects on CGA is unclear. Prior observations reported an increase in phenylpropanoid products and several-fold induction of expression of phenylpropanoid pathway genes in transgenic *p1*-expressing cultures (Grotewold et al., 1998; Bruce et al., 2000), consistent with *p1*'s candidacy for a QTL for CGA synthesis. Additionally, a QTL for CGA in the *p1* region was noticed previously in the (GT114 × GT119)F_2 population (Byrne et al., 1996), and has since been detected in several other populations (Szalma et al., 2002, this issue; Guo et al., 2001).

The *p1* alleles might affect CGA through induction of flavonoid specific genes that increase the flow of substrates toward flavone synthesis, and thus indirectly increase CGA. Within the (A619 × Mp708)F_2 and (A619 × Mo6)F_2 populations functional *p1* alleles increased flavones in an additive manner, but their effects on CGA were dominant (Table 1). This dichotomous gene action might have resulted if flavonoid enzymes reacted in a steady-state manner as substrate flow increased, while the branch toward CGA was quickly saturated from the effects of one *p1* allele. A question remains whether the P1 protein might also induce transcription of genes in the phenylpropanoid pathway. In a (W23 *c2/whp1/in1/p1-www* × Mp708)F_2 population, individuals with functional *p1* alleles showed increased CGA levels in the absence of flavone-producing chalcone synthase activity (Szalma et al., 2002). Since *p1* still affected CGA in the absence of flavone production, its effects on CGA can be separated from flavone synthesis. This is supported by transgenic *p1* induction of the phenylpropanoid gene *pal* (Bruce et al., 2000), which was implicated as the major enzyme affecting CGA in tobacco (Bate et al., 1994). The action of *p1* on CGA accumulation in maize silk likely results from both inducing genes directly involved in CGA synthesis and increasing substrate flow indirectly by induction of flavonoid genes.

The enzyme steps involved in CGA synthesis must be coordinately regulated along with lignins, flavonoids, and other phenylpropanoid branch pathways to match the varying needs of the plant. *Myb*-domain transcription factors C1 and P1 each induce specific flavonoid and anthocyanin genes in maize, and *Myb*-domain gene products in several other plants induced transcription of phenylpropanoid and lignin genes (Martin and Paz-Ares, 1997). The strong effect of *Myb*-domain proteins on target genes is often reflected in QTL analyses, and was seen for *p1* in the current study. Approximately half of all digenic epistatic interactions in the multiple-effects models of the (A619 × Mp708)F_2 and (A619 × Mo6)F_2 populations included the *p1* locus as one of the interacting markers (Table 2), and the other locus invariably depended on functional *p1* alleles to affect the trait (flavone or CGA). Thus, transcription factors should be the first, and most easily detectable, targets in manipulating traits involving pathways (Grotewold et al., 1998).

The *qtl2* Locus

Within bin 2.06, we detected a QTL with large effects on CGA in the (A619 × Mp708)F_2 and (A619 × Mo6)F_2 populations that we named *qtl2*. This QTL was located

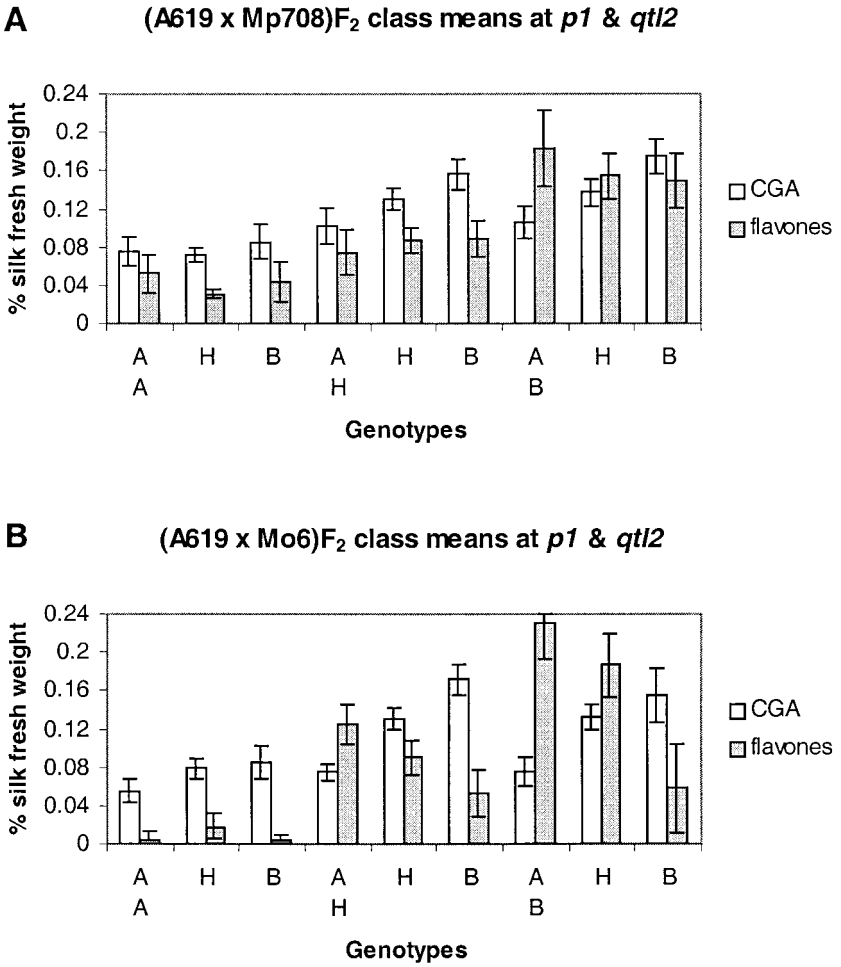

Fig. 4. Genotype class means for CGA and flavones of markers nearest to *p1* and *qtl2* from A: (A619 × Mp708)F_2 and B: (A619 × Mo6)F_2 populations. Genotypes of *p1* are represented by the bottom row of letters, and subdivided into *qtl2* genotypes where A, homozygous A619 alleles; H, heterozygous alleles; and B, homozygous Mp708 or Mo6 alleles dependent upon the population. Standard error bars are taken to the second standard deviation.

near *umc1065* in bin 2.06, and confirmed in other populations utilizing Mp708 as a parent (Szalma et al., 2002). Mo6-derived populations also segregated for a nonfunctional *sm2* allele that was located in bin 2.08, linked to *qtl2*, and identified as a significant locus for flavone synthesis. The *sm2* mutation in Mo6 associates with an accumulation of isoorientin, a C-glucosylflavone lacking the rhamnosyl moiety of maysin (McMullen, unpublished data). The maximum likelihood position of the *sm2* QTL affecting maysin in the (A619 × Mo6)F_2 population was approximately 20 cM distal to the *qtl2* affecting CGA. Additionally, in the (A619 × Mo6)F_2 population a QTL affecting flavone was detected near the putative *qtl2* location, again 20 cM apart from *sm2* (Table 1). In the (Mo6 × Mp708)F_2 population, a QTL was located in bin 2.08 affecting both CGA and flavones. As (Mo6 × Mp708)F_2 likely did not segregate for *qtl2*, but did segregate for *sm2*, the effects at that region were attributed to *sm2*. Thus, although the *sm2* locus was linked to *qtl2*, and slightly affected CGA synthesis in the (Mo6 × Mp708)F_2 population, it was distinct from the gene causing the *qtl2* locus effects.

Mo6 and Mp708 exhibit a rare ability to synthesize more CGA than flavones. This ability is not a result of *p1* function, which is present and segregating in many populations that do not synthesize appreciable amounts of CGA. Rather, it results primarily from the effects of the *qtl2* locus, which to our knowledge has only been detected in lines that synthesize significant amounts of CGA. On the basis of genotype means, *qtl2* had the ability to increase CGA without significant changes in flavones (Fig. 3). However, when *qtl2* class means were subdivided into *p1* alleles, the increase in CGA associated with *qtl2* was concomitant with a decrease in flavones in the presence of a functional *p1* allele classes in the (A619 × Mo6)F_2 population (Fig. 4). This trend was also seen in the (A619 × Mp708)F_2 population. Interestingly, without functional *p1* alleles the effects of the *qtl2* locus on CGA were negligible, and without *qtl2* the effects of *p1* on CGA were negligible. It seems that as the *p1* gene product turns on the phenylpropanoid and flavonoid pathways, *qtl2* diverts substrates toward specific portions of the CGA biosynthetic branch.

As CGA is implicated in host-plant resistance to corn earworm, breeders might be interested in increasing CGA in silks. In many individuals of the (Mo6 ×

Mp708)F_2 population, CGA levels reached an excess of 0.40% of the fresh silk weight. Assuming a comparable activity against corn earworm on the basis of similar chemical structure, this amount of CGA is well above a 0.20% fresh weight threshold of maysin reported to reduce the weight of corn earworm larvae by 50% (Wiseman et al., 1992). Additionally, many individuals from this population synthesized both CGA and flavones, each in amounts well above the 0.20% threshold level. Although these individuals have alleles with equivalent effects at both the *p1* and *qtl2* loci, several novel QTLs were detected in this study with the potential to further increase CGA.

Minor QTLs

Quantitative trait loci for both CGA and flavones were detected that exhibited small effects, and for which candidates were assigned based on location alone. The *pal1* locus was within a confidence interval of a QTL detected in the (A619 × Mo6)F_2 population for CGA. A *c2* candidate locus in bin 4.08 was detected by CIM and the multiple-effects model in the (Mo6 × Mp708)F_2 population for CGA, in which the allele from Mp708 increased CGA. Additionally, an epistatic interaction between the *c2* candidate QTL and a marker in bin 3.04 from the (A619 × Mp708)F_2 population was detected. A QTL for flavones in bin 9.02 was detected in both high-low populations, but only exerted small effects. The *rem1* candidate gene for that QTL was characterized by its ability to increase maysin through recessive inheritance (Byrne et al., 1996, 1998), but in both high-low populations in this study the QTL effects in that region were nearly additive.

The remaining QTLs had no candidate genes that could explain their effects. Among these, there were two to three QTLs detected on chromosome 3 affecting CGA. Indeed, in eight different F_2 populations assayed for CGA accumulation in silks, effects from QTLs in bins 3.03-3.06 were detected (this paper; Szalma et al., 2002; McMullen, unpublished data). As they were detected across different inbred backgrounds and environments, their influence on CGA accumulation in silk tissue is unequivocal. The genetic basis of QTLs for CGA on chromosome 3 warrant further investigation, and specific populations must be established to effectively study their effects.

CONCLUSIONS

Of the QTLs detected affecting CGA biosynthesis, the *p1* and *qtl2* loci had the largest effects in the (A619 × Mp708)F_2 and (A619 × Mo6)F_2 populations. The *p1* locus exhibited additive gene effects on flavone accumulation in silks, but dominant effects on CGA accumulation. The *qtl2* locus main effects increased CGA levels, but in the presence of functional *p1* alleles *qtl2* increased CGA and decreased flavones. In the (Mo6 × Mp708)F_2 population neither locus was segregating and neither QTL was detected, as might be expected if genetic variation at those loci were abolished by similar alleles in the parents. These studies provide candidate genes on which to focus genetic tests to manipulate CGA levels.

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